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Award Number: W81XWH-04-1-0056

TITLE: Comparative Analysis of Vitamin A (Retinol) Regulated Genes in African-American and Caucasian Prostate Cancer Patients

PRINCIPAL INVESTIGATOR: Sue Ellen K. Touma
Lorraine J. Gudas, Ph.D.
David M. Nanus, M.D.
Satish K. Tickoo, M.D.

CONTRACTING ORGANIZATION: Weill Medical College of Cornell University
New York, New York 10021-4870

REPORT DATE: March 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2007	Annual Summary (15 Feb 2004 - 14 Feb 2007)

4. TITLE AND SUBTITLE
Comparative Analysis of Vitamin A (Retinol) Regulated Genes in African-American and Caucasian Prostate Cancer Patients

5. FUNDING NUMBERS
W81XWH-04-1-0056

6. AUTHOR(S)
Sue Ellen K. Touma
Lorraine J. Gudas, Ph.D.
David M. Nanus, M.D.
Satish K. Tickoo, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Weill Medical College of Cornell University
New York, New York 10021-4870

E-Mail: Set2002@med.cornell.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Vitamin A (retinol) and its related metabolites like retinoic acid (RA) have great potential in their roles as prostate cancer chemopreventive and chemotherapeutic agents by exerting regulation on cell growth and differentiation. Several studies have shown that there is a reduction in retinoid levels and retinoid receptors (e.g. RARβ2) in prostate cancer. RA is being used to treat patients with prostate cancer and has been shown to inhibit tumor growth and reverse the events of carcinogenesis in animal models of prostate cancer. There is a disparity in prostate cancer among the African-American population and we hypothesize that more severe disruptions of retinoid signaling occur, contributing to this disparity. The purpose of this study is to examine the underlying causes for the clinical behavior of prostate cancer in African-Americans as compared to Caucasian patients. Immunohistochemical analysis has shown the expression of LRAT, an enzyme responsible for retinol esterification and storage as retinyl esters, to be reduced in tumor tissue specimens from prostate cancer patients as compared to adjacent nonmalignant tissue. Understanding the role of retinoid signaling in prostate carcinogenesis will lead to improved chemoprevention strategies and to the development of novel therapies for this disease.

14. SUBJECT TERMS
Retinoids, Vitamin A Metabolism, Prostate Cancer, Caucasian, African-American

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Prostate cancer is the most common cancer in men in the United States. A disparity exists in the African-American male population in the United States. Incidence rates of prostate cancer are about 70% higher than in white males, African-American patients present with more advanced and aggressive disease, and have poorer survival rates (1, 2). A possible cause for this observed disparity is that there are more dramatic differences among known biological risk factors for African-Americans compared to other populations. Retinoids, retinol (vitamin A), and related metabolites like retinoic acid (RA) serve as cancer chemopreventive and chemotherapeutic agents by exerting regulation on cell growth and differentiation (3). Retinoid actions are mediated by binding two different families of nuclear RA receptors, RARs and RXRs, each with α, β, and γ subtypes (4, 5). It has been shown that there is a reduction in the levels of retinoids and retinoid receptors (e.g. RARβ) in prostate cancer (6). Additionally, recent studies from our laboratory have shown that the levels of LRAT (lecithin:retinol acyltransferase), the primary enzyme responsible for the metabolism of retinol to retinyl esters, are reduced in many carcinomas, including oral cavity, skin, breast, bladder, renal and prostate (7-11).

The purpose of this study is to examine the underlying causes for the clinical behavior of prostate cancer in African-Americans as compared to Caucasian patients. I hypothesize that the molecular events, i.e. greater reductions in the expression of retinoid receptors or retinoid regulated genes, such as RARβ or LRAT, involved in the disruption of retinoid signaling in prostate carcinogenesis are more profound in the African-American population. Currently, the expression of selected retinoid receptors and target genes are being evaluated using immunohistochemical methods, utilizing paraffin-embedded sections obtained from African-American and Caucasian prostate cancer patients in a double-blinded fashion. Prostate tissue will also be used to examine protein expression of retinoid-responsive genes. Additionally, the levels of retinol and its metabolites will be measured by reverse phase high pressure liquid chromatography in prostate cancer samples.

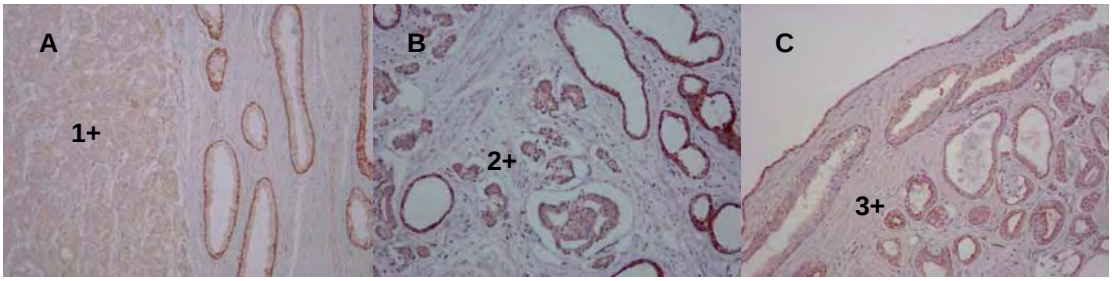
BODY

The first aim of the proposed study is to examine the expression of selected retinoid receptors and retinoid regulated genes in formalin-fixed, paraffin-embedded tissue sections and fresh prostate tumor tissue samples from African-American and Caucasian prostate cancer patients. Previously, LRAT expression was evaluated in 19 paraffin-embedded tumor sections from patients of different races (Caucasian, African-American, Asian, and Hispanic) by immunohistochemical methods as described previously (Figure 1) (10). These prostate tissue specimens were obtained by the Department of Pathology and the Urological Oncology Division at New York Presbyterian Hospital-Weill Cornell Medical Center in a double-blinded fashion. Prior to immunohistochemical staining, hemotoxylin and eosin staining was used to confirm the malignant and benign phenotypes of the specimens. Tissue sections were deparaffinized followed by rehydration in a graded series of ethanol. Antigen retrieval was performed with an antigen unmasking solution in a pressure cooker. The expression of LRAT was determined using an affinity purified polyclonal human LRAT antibody. Incubation with horseradish peroxidase conjugated secondary antibodies was followed by color development using diaminobenzidine (DAB) as substrate and counterstaining with hemotoxylin. As a negative

control, preimmune serum was used in place of primary antibody. LRAT staining was scored in a semiquantitative fashion by Dr. Satish Tickoo, our collaborating pathologist. Specimens were graded according to the intensity of staining within the tumor compared with the intensity of staining of the adjacent, benign tissue. Tumors were classified as 0 (no staining), 1+ (weak staining), 2+ (distinct staining, but weaker than the staining in benign tissue), and 3+ (staining equal to that in the benign tissue).
The expression of LRAT was reduced in tumor tissue specimens from prostate cancer patients as compared to adjacent nonmalignant tissue (Figure 1A). However, there were no detectable trends correlating staining with race or Gleason grade (Figure 1B). This could be due to the fact that the sample size was small.

Our request was declined for additional paraffin-embedded prostate tumor samples from several tissue banks. Although we had included statistical analysis of the pilot study in our application, one point raised from reviewers suggested additional statistical analysis. John Rutledge, a biostatistician from the Clinical Research Methodology core facility at Weill Medical College of Cornell University, utilized odds ratios analysis to determine sample size requirements. Samples classified as 0, 1, or 2 were categorized as having a low level of staining and samples classified as 3 were categorized as having a high level of staining. Odds ratio analysis allows for determining the odds of having a high level of staining increases as the race of the patient changes. Once the desired odds ratio is set, the sample size necessary to achieve it can be calculated (Table 1).

A



B

Sample Number	Race	Gleason Score	Staining
1	A	6	1
2	A	7	1
3	A	7	2
4	A	9	3
5	B	6	3
6	B	7	1
7	B	7	2
8	B	7	2
9	B	7	3
10	B	8	2

Sample Number	Race	Gleason Score	Staining
11	C	6	1
12	C	6	1
13	C	6	3
14	C	7	1
15	C	7	1
16	C	7	2
17	H	6	2
18	H	7	1
19	H	7	2

Figure 1: LRAT expression in human prostate tumor tissue specimens. Paraffin-embedded tissue sections from radical prostatectomy specimens (*n*=19) containing prostate cancer were stained with affinity-purified LRAT antibodies and counterstained with hematoxylin. Negative controls were incubated with preimmune serum instead of primary antibody (not shown). LRAT protein expression is visualized by dark brown staining and is present in basal epithelial cells in areas of normal epithelium. LRAT immunostaining was graded as follows: 1+ (weak staining), 2+ (distinct staining, but weaker than the staining in benign tissue), and 3+ (staining equal to or greater than that in the benign tissue).

A) Representative images for each grade are shown; **A**, 1+, **B**, 2+, and **C**, 3+.

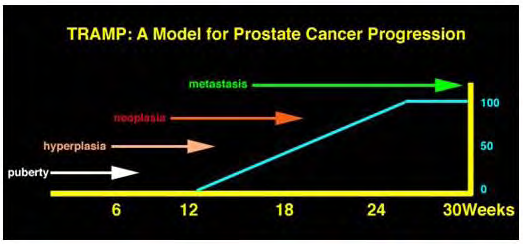
B) Race, Gleason, and staining scores for each patient. **A**, Asian, **B**, Black/African-American, **C**, Caucasian, and **H**, Hispanic.

Table 1: Sample sizes needed to detect various odds ratios

Odds Ratio	Sample Size Needed (per group)
2.74	90
3.03	77
3.30	69
3.61	61
3.99	55
4.20	52
4.43	49

Immunohistochemical analysis will be continued using the existing patient tumor tissue sections, examining the expression of retinoid regulated genes, such as RARβ, RALDH2 (retinaldehyde-specific dehydrogenase type 2), and CYP26A1. Both RALDH2 and the cytochrome p450 enzyme, CYP26A1, are involved in retinoic acid metabolism. These genes, with the exception of CYP26A1, have been found to be reduced in prostate cancer (12-14). Currently, there is a lack of antibodies with adequate specificity for RARβ, RALDH2, and CYP26A1. In order to address this need, we have generated polyclonal antibodies to these three proteins by contract with Alpha Diagnostics International.

Since the last progress report submission, we have tested sera with positive and negative controls from cell and mouse prostate tissue extracts from TRAMP (Transgenic Adenocarcinoma Mouse Prostate) mice to confirm specificity by western blot analysis. Briefly, the TRAMP model was developed by the laboratory of Norman Greenberg (15, 16). It was generated using prostate-specific probasin promoter driving the expression of SV40 large T antigen (Tag) coding region, an oncoprotein that interacts with Rb and p53. Mice expressing high levels of the transgene display progressive forms of disease that histologically resembles human prostate cancer. Tumors have been shown to develop as early as 10 weeks of age, ranging from mild intraepithelial hyperplasia to malignant neoplasia (Figure 2). The pathobiology of prostate cancer in the TRAMP model has been recently characterized including incidence of cancer, metastasis, and expression of differentiation markers (15).

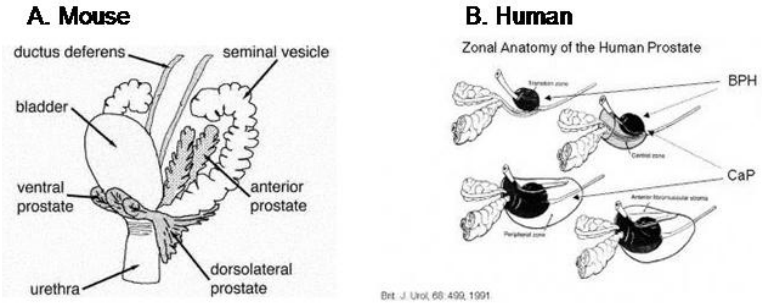


Images courtesy of Greenberg Laboratory website: http://thegreenberglab.fhcrc.org/

Figure 2: Timeline of prostate cancer progression in the TRAMP model.

Retinoid signaling and metabolism have not been thoroughly examined in the TRAMP model and there is potential for characterization at different stages of prostate carcinogenesis. We decided to use this model in these studies for two reasons. First, the unavailability of human prostate tumor tissue for this study was an obstacle in completing the proposed experiments. Secondly, the range of disease states exhibited in this mouse model allows us to correlate cancer in the mouse to prostatic intraepithelial neoplasia (PIN) and tumors of different Gleason scores found in human prostate cancer. We are confident that any molecular changes we observe in this mouse model give us insight into what is happening in human disease. This will further our understanding of prostate cancer and can identify relevant gene and protein targets to examine in human prostate tumor specimens. With these results, we are able to focus specifically on potential molecular markers involved in how racial differences affect the clinical behavior of prostate cancer.

The anatomy of the mouse prostate differs from the human prostate (Figure 3). The mouse prostate consist of four lobes: ventral (VP), lateral (LP), dorsal (DP), and anterior (AP), while the human prostate is divided into three sections including the central, peripheral and transitional zones. Benign prostatic hyperplasia (BPH) tends to occur in the transition zone of the prostate while most cancers arise in the peripheral zone (17). In the TRAMP model, the majority of cancers develop in the dorsal/lateral lobes, which is considered to be most analogous to the peripheral zone in human prostate (18).



Images courtesy of http://emice.nci.nih.gov/mouse_models/organ_models/prostate_models

Figure 3: Anatomy of mouse (A) and human (B) prostate.

To date, I have dissected out prostate tissue from transgenic positive and negative mice and looked at the expression of LRAT, RALDH2, and RARβ by western blot analysis. Mice were dissected at different time points, ranging from 12-36 weeks old. LRAT expression was detected only in the ventral prostate at 12 and 24 weeks (Figure 3). Expression was lower in the

TRAMP+ samples as compared to the TRAMP- prostate samples, with an absence of LRAT by 24 weeks of age in the transgenic positive animal. Protein extracts from COS-7 cells overexpressing mouse LRAT were used as a positive control for antibody specificity. These results are consistent with the reduced LRAT levels observed in the human prostate tumor specimens as compared to adjacent normal tissue (Figure 1A). Two isoforms of RALDH2 are expressed in the prostate (Figure 3). A 49 kilodalton protein expressed in the lateral prostates of transgenic *positive* mice and lost in transgenic *negative* mice and a 35 kilodalton protein expressed in the dorsal prostates of transgenic *negative* mice and lost in transgenic *positive* mice. Tissue extracts of testis from a wild type male mouse was used as a positive control for this antibody. The significance of the differential expression of RALDH2 within the mouse prostate is still unclear but we are pursuing this finding since the expression of RALDH2 is known to be lost in human prostate cancer as well (14). RARβ expression was detected only in the dorsal and anterior lobes of mouse prostate (Figure 4). More importantly, RARβ is expressed only in the transgenic *negative* samples at 18 and 30 weeks and is absent in the transgenic *positive* counterparts. Both COS-7 mock and COS-7 cells overexpressing mouse LRAT served as negative controls for this antibody. Protein extracts from COS-7 cells overexpressing human RARβ were loaded as a positive control for this antibody. Both RALDH2 and RARβ antibodies cross react with both mouse and human isoforms, so these antibodies can be used in future studies with human tissue. We are currently staining patient tumor tissue sections with these antibodies and will perform similar analysis as done with the LRAT study.

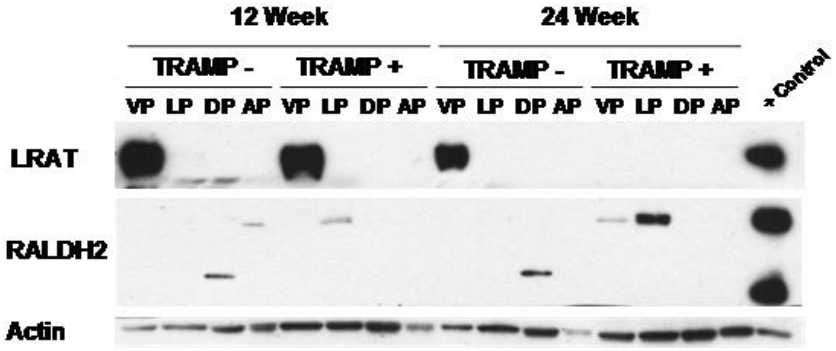


Figure 3: LRAT and RALDH2 expression in the mouse prostate. Prostate lobes were dissected, homogenized in SDS sample buffer, 20 µg protein/sample were separated on a 12% SDS-PAGE gel, and transferred to nitrocellulose membranes. LRAT and RALDH2 expression were examined using affinity-purified, polyclonal rabbit antimouse LRAT antibody (1:500). Immunoblot analysis of actin (1:400, Santa Cruz Biotechnology, Santa Cruz, CA) served as a loading control. Primary antibody incubation was done overnight at 4°C. Blots were incubated with an IgG horseradish peroxidase–conjugated secondary antibody at room temperature (anti-rabbit for LRAT and RALDH2, 1:5,000 dilution; anti-goat for actin, 1:2,000 dilution; Santa Cruz Biotechnology). *VP:* ventral prostate, *LP:* lateral prostate, *DP:* dorsal prostate, *AP:* anterior prostate. *Positive controls:* LRAT-Whole cell extract of COS-7 cell line overexpressing mouse LRAT; RALDH2-testis tissue extract from a wild type mouse.

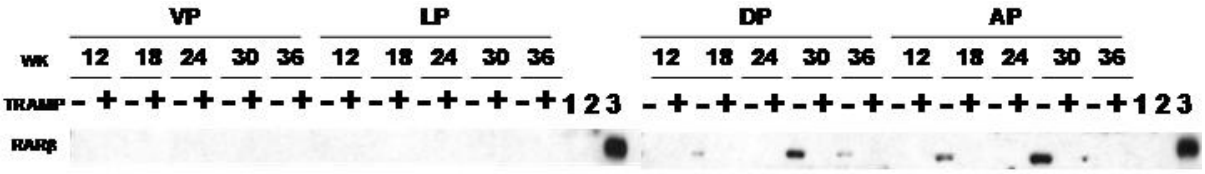


Figure 4: LRAT and RARβ in the mouse prostate. Prostate lobes were dissected, homogenized in SDS sample buffer, 15 µg protein/sample were separated on a 15% SDS-PAGE gel, and transferred to nitrocellulose membranes. The membranes were stained with Ponceau S (Sigma) to confirm proper transfer and equal loading. Immunoblot analysis was performed using affinity-purified, polyclonal rabbit anti-mouse antibodies (1:500). Primary antibody was incubated overnight at 4°C. Blots were incubated with an IgG horseradish peroxidase–conjugated secondary

antibody at room temperature (anti-rabbit, 1:5,000 dilution; Santa Cruz Biotechnology). *VP:* ventral prostate, *LP:* lateral prostate, *DP:* dorsal prostate, *AP:* anterior prostate. *1:* Whole cell extract of COS-7 cell line, *2:* Whole cell extract of COS-7 cell line overexpressing mouse LRAT, *3:* Whole cell extract of COS-7 cell line overexpressing human RARβ.

Additionally, prostate, lung, and liver tissue from wild type, transgenic negative, and transgenic positive mice were excised and retinoid levels were measured using HPLC methods. Following tissue homogenization, retinoids were extracted using acetonitrile/butanol and retinoids were assayed and measured by reverse phase high pressure liquid chromatography linked to a photodiode array detector. Preliminary results have shown that retinol and retinyl ester levels in TRAMP positive mice are lower than transgenic negative littermate and wild type controls (Figure 5). Lung and liver tissues have high retinoid levels and served as positive controls for this experiment. There are no significant changes in both retinol and retinyl ester levels among all samples tested.

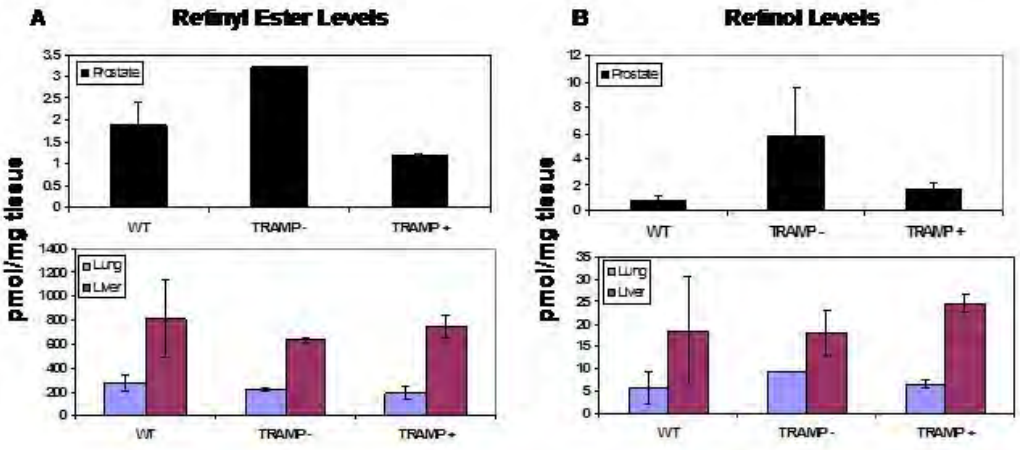


Figure 5: Levels of retinyl esters and retinol in prostate, lung and liver tissue. Retinoids were extracted from prostate, lung, and liver tissues from 28 week old TRAMP + and – mice (2 of each) and 24 week old WT mice (4) and separated by reverse-phase HPLC analysis. *Bars*, SD.

KEY RESEARCH ACCOMPLISHMENTS

- Learned how to stain paraffin-embedded tumor tissue sections using immunohistochemical methods.
- Completed a pilot study looking at LRAT staining in prostate cancer patients of different races and obtained the necessary statistical analysis to move ahead with this project.
- Learned strategies for optimal antigenic peptide design for antibody production as well as antibody purification by affinity chromatography methods.
- Utilized a mouse model of prostate cancer (TRAMP) to continue studies as well as testing custom made antibodies for this fellowship project.
- Learned to extract retinoids from prostate samples and use HPLC methods to measure tissue retinoid levels.

REPORTABLE OUTCOMES

Poster Presentation
American Association for Cancer Research Third Annual International Conference on Frontiers in Cancer Prevention Research, Seattle, WA, October 16-20, 2004
Title: Analysis of Retinoid Signaling and Metabolism in Prostate Cancer.
Authors: Sue Ellen Touma, Satish K. Tickoo, David M. Nanus, Dean Bok, and Lorraine J. Gudas

Poster Presentation
American Association for Cancer Research Special Conference: Mouse Models of Cancer, Cambridge, MA, October 25-28, 2006
Title: Analysis of Retinoid Signaling and Metabolism in Prostate Cancer.
Authors: Sue Ellen Touma, Satish K. Tickoo, David M. Nanus, Dean Bok, and Lorraine J. Gudas

CONCLUSIONS

This proposal addresses fundamental aspects of retinoid signaling and prostate cancer biology. We have shown that there is a decrease in LRAT expression in tumor tissue as compared to the adjacent benign tissue in prostate tumor samples. We must increase our sample size to determine if we can further distinguish differences in LRAT staining based on race and/or Gleason grade. While we are pursuing resources for additional prostate tissue for this study, we have been putting our efforts towards investigating other retinoid regulated targets (RARβ, ALDH1a2, and CYP26A1) involved in retinoid signaling and metabolism that are known to be downregulated in prostate cancer. Specific antibodies were not available for RARβ, ALDH1a2, and CYP26A1. To this end, we have generated polyclonal antibodies against RARβ, ALDH1a2, and CYP26A1 for use in staining tumor tissue. We used the TRAMP model of prostate cancer to conduct experiments alongside patient tissue. The use of the TRAMP model allows for a more thorough molecular and pharmacological characterization of retinoid signaling pathways during the process of prostate carcinogenesis. To date, we have shown that LRAT, RALDH2, and RARβ expression is altered in TRAMP positive mice. Additionally, both retinyl ester and retinol levels are reduced in TRAMP positive mice. Together, the analysis of patient tumor specimens along with the TRAMP model will ultimately lead to improved chemoprevention strategies and to the development of novel therapies for prostate cancer. Differences in retinoid regulated gene expression may account for the observed disparity and serve as markers for enhanced detection of this disease.

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